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EP 0 561 424 B1

Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to liposomes and in particular to dehydrated liposomes which can be stored for extended periods of time and then rehydrated when and where they are to be used.

2. Description of the Prior Art

As is well known in the art, liposomes are closed vesicles having at least one lipid bilayer membrane surrounding an aqueous core. One of the primary uses for liposomes is as carriers for a variety of materials, such as, drugs, cosmetics, diagnostic reagents, bioactive compounds, and the like.

In connection with each of these uses, it is important to be able to store liposomes for long periods of time without substantial leakage from the liposomes of the selected materials they are carrying. More particularly, so as to be useful in commercial settings, liposome preparations must have long enough shelf-lives to allow them to be easily manufactured, shipped, and stored by intermediate and ultimate users under a variety of temperature conditions.

With particular regard to the drug industry, it is also important to be able to provide drug manufacturers with unloaded liposomes which the manufacturers can subsequently load in their own plants with their own drugs. Such a two step or two factory approach (i.e., manufacturing unloaded liposomes in a first plant and then filling them in a second plant) would allow drug manufacturers to purchase a defined commodity, i.e., unloaded liposomes, from suppliers and then use that commodity as an off-the-shelf component of their final product.

As drug manufacturers currently operate their businesses, they strongly prefer to buy defined commodities from suppliers and then assemble the final product in their own plants. In this way, they can personally control the quality of the finished products. Usage of liposome technology by the drug industry would be greatly enhanced if liposomes could also be provided to manufacturers as a defined commodity.

To date, liposome preparations have generally had relatively short shelf-lives. Moreover, there has been no known way to prepare liposomes at one point in time and then fill them with selected materials at a much later point in time. The present invention makes up for these existing shortcomings in the current state of the art.

SUMMARY OF THE INVENTION

In view of the above state of the art, it is an object of the present invention to provide liposome preparations which can be stored for extended periods of time without substantial leakage from the liposomes of internally encapsulated materials.

It is a further object of the present invention to provide liposome preparations which can be dehydrated, stored for extended periods of time while dehydrated, and then rehydrated when and where they are to be used, without losing a substantial portion of their content during the dehydration, storage and rehydration processes.

It is an additional object of the present invention to provide liposome preparations which can be dehydrated, stored for extended periods of time while dehydrated, rehydrated, and then filled with selected materials.

To achieve these and other objects, the invention, in accordance with one of its aspects, provides a method for dehydrating liposomes as claimed in claims 1 to 7 and liposome preparations as claimed in claims 8 to 12. In certain embodiments of the invention, the liposomes are dehydrated with one or more sugars being present at both the inside and outside surfaces of the liposome membranes. In other embodiments, the sugars are selected from the group consisting of trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose.

The dehydration is done under vacuum and take place without prior freezing of the liposome preparation.

When done without prior freezing, use of the protective sugars can be omitted when (1) the liposomes being dehydrated are of the type which have multiple lipid layers, and (2) the dehydration is done to an end point where there is sufficient water left in the preparation so that a substantial portion of the membranes retain their integrity upon rehydration. Preferably, at lease about 2%, and most preferably between about 2% and about 5%, of the original water in the preparation prior to dehydration should remain in the preparation at the end of the dehydration process. In terms of moles of water per mole of lipid in the dehydrated preparation, this corresponds to a water level of preferably at least about 12 moles water/mole lipid, and most preferably between about 12 and about 35 moles water/mole lipid, in the dehydrated preparation.

In accordance with other aspects of the invention, delayed loading of preformed liposomes is achieved by creating a concentration gradient across the liposome membranes of one or more charged species which, under suitable conditions, are capable of passing across those membranes. This concentration gradient is used to load selected charged

materials, e.g., drugs, into the liposomes through the creation of a transmembrane potential.

It has been found that liposomes having a concentration gradient across their membranes can be dehydrated in the presence of one or more sugars, as described above and in more detail below, stored in their dehydrated condition, subsequently rehydrated, and the concentration gradient then used to create a transmembrane potential which will load charged materials into the liposomes. Alternatively, the concentration gradient can be created after the liposomes have been dehydrated, stored, and rehydrated. Also, if the dehydration is done without prior freezing of the liposomes and under the conditions described above, the use of protective sugars may be omitted.

The attainment of the foregoing and other objects and advantages of the present invention is described fully below in connection with the description of the preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the retention of inulin in freeze and thaw multilamellar vesicles (FATMLVs) and stable plurilamellar vesicles (SPLVs) as a function of the percentage of the original water remaining in the preparation at the end of the dehydration process. The liposomes were dehydrated in the absence of protective sugars under reduced pressure.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above, the present invention relates to liposomes which can be subjected to long-term storage without substantial loss of their internal contents. The liposomes are stored in a dehydrated state. The liposomes being dehydrated are of the type which have multiple lipid layers and the dehydration is performed without prior freezing and to an end point where is sufficient water left in the preparation so that a substantial portion of the membranes retain their integrity upon rehydration, the use of a protective sugar may be omitted.

The liposomes which are to be dehydrated can have a variety of compositions and internal contents, and are in the form of multilamellar, stable plurilamellar liposomes (SPLVs), monophasic vesicles (MPVs), or lipid matrix carriers (LMCs) of the types disclosed in copending and commonly assigned U.S. Patent Nos. 4,522,803, 4,588,578, 4,610,868 and 4,721,612, filed March 24, 1983, August 8, 1983, March 20, 1984, and April 12, 1984, respectively, or can be in the form of freeze and thaw multilamellar vesicles (FATMLVs) of the type described in copending and commonly assigned US-A-4,975 282, filed July 5, 1985, and entitled "Multilamellar Liposomes Having Improved Trapping Efficiencies,".

The liposomes can be prepared by any of the techniques now known for preparing liposomes. For example, the liposomes can be formed by the conventional technique for preparing multilamellar liposomes (MLVs), that is, by depositing one or more selected lipids on the inside walls of a suitable vessel by dissolving the lipids in chloroform and then evaporating the chloroform, adding the aqueous solution which is to be encapsulated to the vessel, allowing the aqueous solution to hydrate the lipid, and swirling or vortexing the resulting lipid suspension to produce the desired liposomes.

A review of methods for producing liposomes can be found in the text Liposomes, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1. See also Szoka, Jr., et al., (1980) Ann. Rev. Biophys. Bioengr., 9:467.

As other alternatives, the liposomes can be produced in accordance with the procedures described in US-A-4,522,803, US 4,588,578, US-A-472,612 referred to above, or in accordance with the freeze and thaw procedures described in US-A-4 975 282 also referred to above. Also, rather than using liposomes per se, other lipid-containing particles, such as those described in U.S. Patent US-A-4,610,868 referred, to above, can be used in the practice of the present invention.

The liposomes are preferably dehydrated using standard freeze-drying equipment or equivalent apparatus, that is, they are preferably dehydrated under reduced pressure. If desired, the liposomes and their surrounding medium can be frozen in liquid nitrogen before being dehydrated. Alternatively, and quite surprisingly, the liposomes can also be dehydrated without prior freezing, by simply being placed under reduced pressure. Dehydration without prior freezing takes longer than dehydration with prior freezing, but the overall process is gentler without the freezing step, and thus there is in general less damage to the liposomes and a corresponding smaller loss of the internal contents of the liposomes. For example, dehydration without prior freezing at room temperature and at a reduced pressure provided by a vacuum pump capable of producing a pressure on the order of 1 mm of mercury can take between approximately 24 and 36 hours, while dehydration with prior freezing under the same conditions can take between approximately 12 and 24 hours.

So that the liposomes will survive the dehydration process without losing a substantial portion of their internal contents, it is important that one or more protective sugars be available to interact with the liposome membranes and keep them intact as the water in the system is removed. A variety of sugars can be used, including such sugars as trehalose, maltose, sucrose, glucose, lactose, and dextran. In general, disaccharide sugars have been found to work better than monosaccharide sugars, with the disaccharide sugars trehalose and sucrose being most effective. Other more complicated sugars can also be used. For example, aminoglycosides, including streptomycin and dihydrostreptomycin, have been found to protect liposomes during dehydration.

The one or more sugars are included as part of either the internal or external media of the liposomes. Most preferably, the sugars are included in both the internal and external media so that they can interact with both the inside and outside surfaces of the liposomes' membranes. Inclusion in the internal medium is accomplished by adding the sugar or sugars to the solute which the liposomes are to encapsulate. Since in most cases this solute also forms the bathing medium for the finished liposomes, inclusion of the sugars in the solute also makes them part of the external medium. Of course, if an external medium other than the original solute is used, e.g., to create a transmembrane potential (see below), the new external medium should also include one or more of the protective sugars.

The amount of sugar to be used depends on the type of sugar used and the characteristics of the liposomes to be protected. Persons skilled in the art can test various sugar types and concentrations to determine which combination works best for a particular liposome preparation. In general, sugar concentrations on the order of 100 mM and above have been found necessary to achieve the highest levels of protection. In terms of moles of membrane phospholipid, millimolar levels on the order of 100 mM correspond to approximately 5 moles of sugar per mole of phospholipid.

In the case of dehydration without prior freezing, if the liposomes being dehydrated are of the type which have multiple lipid layers and if the dehydration is carried out to an end point where there is sufficient water left in the preparation so that a substantial portion of the membranes retain their integrity upon rehydration, the use of one or more protective sugars may be omitted. As discussed above, it has been found preferable if the preparation contains at the end of the dehydration process at least about 2%, and most preferably between about 2% and about 5%, of the original water present in the preparation prior to dehydration.

Once the liposomes have been dehydrated, they can be stored for extended periods of time until they are to be used. The appropriate temperature for storage will depend on the make up of the liposomes and the temperature sensitivity of the encapsulated materials. For example, as is well known in the art, various drug preparations are heat labile, and thus dehydrated liposomes containing such drugs should be stored under refrigerated conditions so that the drugs do not lose their potency. Also, for such drugs, the dehydration process is preferably carried out at reduced temperatures, rather than at room temperature.

When the dehydrated liposomes are to be used, rehydration is accomplished by simply adding an aqueous solution, e.g., distilled water, to the liposomes and allowing them to rehydrate. The liposomes can be resuspended into the aqueous solution by gentle swirling of the solution, The rehydration can be performed at room temperature or at other temperatures appropriate to the composition of the liposomes and their internal contents.

The present invention will be more fully described by the following examples. The materials and methods which are common to the various examples are as follows.

Example 1

Dehydration of Liposomes Having Multiple Lipid Layers Without Prior Freezing and Without the Use of a Protective Sugar

This example illustrates that liposomes having multiple lipid layers will retain a substantial portion of their internal contents during dehydration and subsequent rehydration, even without the use of a protective sugar, provided that the dehydration is performed without prior freezing of the liposomes and provided that the dehydration is performed to an end point where there is sufficient water left in the preparation so that a substantial portion of the membranes retain their integrity upon rehydration.

The experiments were performed using the following types of liposomes, all of which include multiple lipid layers: multilamellar liposomes (MLVs), stable plurilamellar liposomes (SPLVs), and monophasic vesicles (MPVs). A detailed description of suitable techniques for producing SPLVs and MPVs appears in copending and commonly assigned U.S. Patent Nos. 4,522,803 and 4,588,578 respectively. Descriptions of methods for preparing MLVs can be found throughout the literature, including, for example, the Liposome text (Marc J. Ostro, ed., 1983) and the Szoka, Jr., et al. reference (Ann. Rev. Biophys. Bioengr., 1980), referred to above.

The materials and experimental protocol used were as follows. All three types of liposomes were made with egg phosphatidylcholine (EPC) obtained from Sigma Chemical Company and with Hepes buffer with and without trehalose (i.e., 20 mM Hepes, 150 mM NaCl, pH 7.4 -- Buffer 0; or 20 mM Hepes, 150 mM NaCl, 250 mM trehalose, pH 7.4 -- Buffer 250). $^{51}CrO_4^=$ (New England Nuclear) in normal saline was used as the tracer. 0.01 ml of the tracer produced a cpm level of approximately 500,000.

For each of the three types of liposomes, the EPC was dissolved in $CHCl_3$ (100 mg/ml) and 3.14 ml of the resulting solution was deposited on the sides of a glass, round bottom flask using a Rotovap evaporator. To make SPLVs, the lipid was redissolved in ether (10 ml) to which was added 0.5 ml of either Buffer 0 or Buffer 250 which included 0.01 ml of the tracer solution. The ether was blown off under a stream of nitrogen while sonicating in a bath sonicator. 4.5 ml of Buffer 0 or Buffer 250 was then added, producing a final lipid concentration of 62.8 mg/ml.

To make MPVs, the lipid was redissolved in 100% ethanol (10 ml) to which was added 0.5 ml of either Buffer 0 or Buffer 250 which included 0.01 ml of the tracer solution. The ethanol was evaporated off at 55-60°C using a Rotovap

evaporator. 4.5 ml of Buffer 0 or Buffer 250 was then added, producing a final lipid concentration of 62.8 mg/ml.

MLVs were made by adding 5.0 ml of Buffer 0 or Buffer 250 to the round bottom flask and then vortexing the samples with glass beads for approximately 5 minutes. The buffers included 0.01 ml of the tracer solution. As with the SPLVs and the MPVs, the final lipid concentration was 62.8 mg/ml.

Once all six liposomes samples had been prepared (one with and one without trehalose for each of the three types of liposomes), they were removed from the round bottom flasks by vortexing for approximately 5 minutes, and each sample was placed in a dialysis bag made of Thomas 12,000 M.W. dialysis tubing. The radioactivity of each bag was counted and the bags were then dialyzed against 500 ml of Buffer 0 or Buffer 250, as appropriate, until a stable count was reached indicating that the tracer had been removed from the external medium surrounding the liposomes. Dialysis for approximately 24 hours was found sufficient to reach a stable count.

Without prior freezing, 1.0 milliliter of each sample was dried for 24 hours in a 10 ml Kimex tube at room temperature under high vacuum using a Virtis Freeze Drier (Gardiner, N.Y.). As the results presented below for the liposome preparations which did not include trehalose show, dehydration for this period of time and under these conditions resulted in dehydrated preparations which included sufficient residual water so that a substantial portion of the liposome membranes retained their integrity upon rehydration even though a protective sugar was not used.

After dehydration, the liposomes were placed in 0.9 ml of distilled water and slowly rehydrated with gentle swirling or vortexing, as necessary.

The rehydrated liposomes were transferred to dialysis bags of the type described above and their radioactivity measured. The bags were then dialyzed against Buffer 0 or Buffer 250, as appropriate, for approximately 18 hours and their radioactivity measured again. The amount of radioactivity retained by the liposomes after dialysis was used as a measure of the amount of internal contents which the liposomes were able to retain through the dehydration/rehydration process. As a control, 1.0 milliliter of each sample was placed in a 10 ml Kimex tube, held at room temperature without drying for 24 hours, placed in a dialysis bag, measured for radioactivity, dialyzed against the appropriate buffer, and then remeasured for radioactivity.

The results of these experiments were as follows, where the percentages given are the counts per minute after dialysis relative to the counts per minute before dialysis and where the numbers in parentheses are the control values:

	0 Buffer	250 Buffer
MLVs	91.9% (87.7%)	84.4% (100.1%)
SPLVs	85.1% (82.7%)	84.3% (94.2%)
MPVs	85.5% (90.1%)	75.9% (93.2%)

As shown by these results, well over 80% of the internal contents of each of the three types of liposomes was retained after the dehydration/rehydration process without the use of any protective sugars. Moreover, adding trehalose to these types or liposomes somewhat decreased, rather than increased, the amount of internal contents retained in the liposomes after the dehydration/rehydration process.

Example 2

Dehydration of Liposomes Without The Use of a Protective Sugar: Quantification of Preferred Residual Water Levels

This example illustrates that when a liposome preparation is dehydrated without the use of a protective sugar, at least about 2%, and preferably between about 2% and about 5%, of the original water in the preparation should remain in the preparation at the end of the dehydration process so that the liposomes will retain a substantial portion of their internal contents upon rehydration.

The experiments were performed using stable plurilamellar liposomes (SPLVs) and freeze and thaw multilamellar vesicles (FATMLVs), both of which include multiple lipid layers. A detailed description of suitable techniques for producing SPLVs appears in U.S. Patent Application 4,522,803 referred to above. See also, Grunner et al., (1985) Biochemistry, 24:2833. A description of techniques for producing FATMLVs can be found in copending and commonly assigned US-A-4 975 282, filed July 5, 1985, and entitled "Multilamellar Liposomes Having Improved Trapping Efficiencies,".

The materials and experimental protocol used were as follows. Egg phosphatidylcholine (99%) was purchased from Avanti Polar Lipids, Inc. (Birmingham, Alabama). [^{14}C]inulin and tritiated water were obtained from New England Nuclear (Boston, Massachusetts). Tritiated water and [^{14}C]inulin were counted in a Beckman LS6800 liquid scintillation counter set for 2 channel operation. All data were corrected for isotope counting efficiency and channel spillover.

SPLVs were prepared by adding 400 umoles of egg PC in chloroform to a 100 ml round bottom flask. Bulk solvent

was removed by evaporation for approximately 2 minutes using a Rotovap evaporator; the lipid was not taken to dryness. Ten milliliters of anhydrous diethyl ether (J.T. Baker Chemical Co., Phillipsburg, New Jersey) was added to the flask to redissolve the lipid. To this solution was added 0.3 ml of equimolar 145 mM NaCl/KCl with 20 mM HEPES (pH 7.4) containing [^{14}C]inulin (16.67 uCi/ml, specific activity 2.4 mCi/g) and unlabeled inulin to bring the final inulin concentration to 1.42 umol inulin/0.3 ml buffer. The samples were sonicated for 1 minute for dispersion and then dried with N_2 while sonicating until the odor of ether was no longer detectable. The lipid was resuspended in 10 ml of buffer and transferred to a 30 ml Corex tube.

Unentrapped [^{14}C] inulin was removed by 4 wash/centrifugation cycles, the centrifugation being conducted for 30 minutes at 12,100 x g in a Beckman J2-21 centrifuge with a JA-20 rotor. The first wash was performed with 10 ml, and subsequent washes with 20 ml, of buffer.

After decanting the final wash supernatant, the vesicle pellet was resuspended with 5 ml of buffer containing tritiated water (2.5 uCi/ml, specific activity 1 mCi/g). The lipid concentration of this preparation was determined by dividing the amount of lipid used (400 umol) by the measured volume of the preparation. For the SPLV liposomes, the average volume was 6.01 ± 0.04 ml, giving an average lipid concentration of 6.65 x 10^{-5} moles lipid/ml.

The radioactivity of the tritiated buffer used to resuspend the vesicles was measured and found to be 5.55 x 10^6 dpm/ml (dpm = disintegrations per minute). To be able to calculate residual water values in the dehydrated preparations in terms of moles of water per mole of lipid (see below), this dpm/ml value was converted to a moles H_2O/dpm value by dividing the dpm/ml value by the concentration of water in the buffer. For this purpose, the buffer was assumed to be pure water so that the concentration of water was 5.55 x 10^{-2} moles H_2O/ml. The moles H_2O/dpm value was thus calculated to be 1.00 x 10^{-8} moles H_2O/dpm.

After resuspension in the tritiated buffer, the preparation was held for a period of at least 30-60 minutes at room temperature prior to dehydration to allow the tritiated water to equilibrate throughout the preparation.

FATMLVs were prepared by adding 400 umoles of egg PC in chloroform to a 100 ml round bottom flask. Solvent was removed by rotary evaporation for 5 minutes, followed by 2 hours under high vacuum in a dessicator (see dehydration discussion below for a description of the specific equipment used).

The lipid was hydrated with 5 ml of equimolar 145 mM NaCl/KCl and 20 mM Hepes (pH 7.4) containing [^{14}C]inulin (1 uCi/ml, specific activity 2.4 mCi/g) and unlabeled inulin to bring the final inulin concentration to 1.08 mM.

The mixture was dispersed by vortexing and aliquots were transferred to 1.8 ml Nunc cryo tubes (Nunc, Denmark). The samples were successively frozen in liquid nitrogen and thawed in warm water five times. The contents were pooled, mixed and transferred to a 30 ml Corex tube.

Unencapsulated [^{14}C]inulin was removed by 4 wash/centrifugation cycles, using 20 ml of buffer for each wash. The centrifugations were performed in the manner described above for SPLVs.

After decanting the final wash supernatant, the vesicle pellet was resuspended with 5 ml of the same tritiated water buffer used to resuspend the SPLVs. The final preparation was held for a period of at least 30-60 minutes prior to dehydration to allow the tritiated water to equilibrate throughout the preparation. As with the SPLV experiments, the lipid concentration of the preparation was determined by dividing the amount of lipid used (400 umol) by the measured volume of the preparation. In this case, the average volume was 7.13 ± 0.06 ml, giving an average lipid concentration of 5.60 x 10^{-5} moles lipid/ml.

The radioactivity due to tritiated water of aliquots of the resuspended SPLVs and FATMLVs was measured and an average value of 4.18 x 10^6 ± 1.49 x 10^5 dpm/ml was obtained for the SPLV suspensions and an average value of 3.60 x 10^6 ± 1.41 x 10^5 dpm/ml was obtained for the FATMLV suspensions. Using the 1.00 x 10^{-8} moles H_2O/dpm value measured for the buffer without vesicles, these radioactivity values were converted to water concentrations for the SPLV and FATMLV suspensions. Specifically, a water concentration of 4.18 x 10^{-2} moles H_2O/ml was calculated for the SPLV suspension, and a water concentration of 3.60 x 10^{-2} moles H_2O/ml was calculated for the FATMLV suspension. As described below, these values along with the lipid concentrations given above were used to calculate the residual water values in the dehydrated preparation in terms of moles of water per mole of lipid.

In addition to measuring the radioactivity of the resuspended preparations due to tritiated water, the radioactivity due to [^{14}C]inulin was also measured,

The preparations were then dehydrated. Specifically, multiple samples were pipetted into 30 ml Corex tubes (approximately 1 ml of suspension per tube), and the weight of the tube plus suspension recorded. The samples were then dried at room temperature under high vacuum with a model D4A Maxima Vacuum Pump (Fisher Scientific, Fairlawn, N.J.) having an ultimate partial pressure rating of 3 x 10^{-4} Torr and a displacement capacity of 127 liters/minute.

The dehydration was carried out for periods of time up to 48 hours, with samples being removed at various points in time and rehydrated with distilled water to their pre-dehydration weight. The vesicles were dispersed by gentle vortexing and the sample was held for approximately 15-30 minutes to allow the tritiated water remaining in the sample after the dehydration process to equilibrate throughout the preparation.

An aliquot was then removed from the sample and its radioactivity per ml due to tritiated water was measured. Percent residual water levels for the dehydrated samples, i.e., the percentage of the original water remaining in the sample after the dehydration process, were then calculated by simply dividing the measured radioactivity levels after rehydra-

tion by the average pre-dehydration values given above, i.e., by 4.18 x 10^6 dpm/ml for SPLVs and 3.60 x 10^6 dpm/ml for FATMLVs.

The percent residual water values were converted to moles of water per mole of lipid in the dehydrated preparation by multiplying the percent values by the water concentrations given above, i.e., by 4.18 x 10^{-2} moles H_2O/ml for SPLVs and by 3.60 x 10^{-2} moles H_2O/ml for FATMLVs, and dividing by the lipid concentrations, i.e., by 6.65 x 10^{-5} moles lipid/ml for SPLVs and 5.60 x 10^{-5} moles lipid/ml for FATMLVs. For example, the calculated values obtained for the integer percentages between 1 and 6 percent were:

% residual water	water/lipid ratio	
	SPLV	FATMLV
6.0	37.7/1	38.6/1
5.0	31.4/1	32.1/1
4.0	25.2/1	25.7/1
3.0	18.9/1	19.3/1
2.0	12.6/1	12.9/1
1.0	6.3/1	6.4/1

After the radioactivity of the rehydrated preparation due to tritiated water had been measured, inulin retention was determined by first subjecting the rehydrated sample to 3 wash/centrifugation cycles using 10 milliliters of buffer per wash. The centrifugation was performed for 25 minutes at 12,100 x g using the equipment described above. The vesicles in the final wash pellet were resuspended to their original weight with buffer and assayed for [^{14}C]inulin. Percent inulin retained values were calculated by dividing the post-rehydration radioactivity values by the pre-dehydration values.

The results of these experiments are shown graphically in Figure 1 As shown therein, the percent inulin retained values remain relatively constant down to a residual water level of about 5%, i.e., a moles of water/moles of lipid value for the dehydrated preparation on the order of 35. Thereafter, increasingly greater amounts of inulin loss are seen with reduced residual water levels, with losses on the order of 30-40% and more being seen once the residual water level drops below about 2.0%, i.e., a moles of water/moles of lipid value for the dehydrated preparation on the order of 12.

Since for long-term storage, it is in general desirable to have a minimal amount of water in the preparation, these results demonstrate that to achieve this goal and still have reasonably low levels of vesicle rupture, the residual water level in the dehydrated preparation should preferably be kept between about 2% and about 5%, or in terms of moles of water per mole of lipid, between about 12 moles H_2O/mole lipid and about 35 moles H_2O/mole lipid.

Example 3

Dehydration of Liposomes Without The Use of a Protective Sugar: Effects of Vesicle Type, Lipid Type and Lipid Concentration

This example illustrates the effects of vesicle type, lipid type, and lipid concentration on the dehydration of liposomes without a protective sugar.

SPLVs and FATMLVs were prepared as described in Example 8 with the following changes: 1) both egg PC and soy PC (Avanti Polar Lipids, Birmingham, Alabama) were used; 2) the starting amount of lipid was either 400 umoles, as in Example 2 or 15 umoles; 3) 1.0 uCi/ml tritiated water was added to the buffer, rather than 2.5 uCi/ml tritiated water; and 4) in the case of SPLVs, inulin and radioactive inulin were added both in accordance with the procedures of Example 2 and by adding [^{14}C]inulin (3.34 uCi/ml, specific activity 2.4 mCi/g) and unlabelled inulin to bring the final inulin concentration to 1.08 umol inulin/0.3 ml buffer. With regard to this last change, it was found that the measured values of retained inulin were indistinguishable for the two radioactive inulin preparations.

MPVs were prepared following the procedures of U.S. Patent No. 4,588,578, referred to above in Example 1. Specifically, either 15 or 400 umoles of egg PC or soy PC in chloroform were added to a 100 ml round bottom flask. The solvent was removed by rotary evaporation for 5-10 minutes, followed by 30 minutes under high vacuum using the equipment described in Example 2. Five milliliters of 100% ethanol was added to the flask to resolubilize the lipids. To this solution was added 0.30 ml of equimolar 145 mM NaCl/KCl with 20 mM HEPES (pH 7.4) containing [^{14}C]inulin (16.67 uCi/ml, specific activity 2.4 mCi/g) and unlabeled inulin to bring the final inulin concentration to 1.42 umol inu-

lin/0.3 ml buffer. The contents of the flask were mixed by vortexing, and the mixture was dried to a thin film by rotary evaporation for 5-10 minutes, followed by 30 minutes under high vacuum, again using the equipment of Example 2. The lipid was resuspended in 5 ml of buffer and transferred to a 30 ml Corex tube.

Unincorporated [^{14}C]inulin was removed by 4 wash/centrifugation cycles, using 20 milliliters of buffer for each wash. Centrifugation was performed for 30 minutes as described in Example 2. After decanting the final wash supernatant, the vesicle pellet was resuspended with 5 ml of buffer containing tritiated water (1.0 uCi/ml, specific activity 1 mCi/g). The preparation was then held for a period of at least 30-60 minutes prior to dehydration to allow for equilibration of the tritiated water throughout the preparation.

MLVs were prepared by adding either 15 or 400 umoles of egg PC or soy PC in chloroform to a 100 ml round bottom flask. Solvent was removed by rotary evaporation for 5 minutes, followed by 2 hours under high vacuum, using the equipment of Example 2 The lipid was hydrated with 5 ml of equimolar 145 mM NaCl/KCl with 20 mM HEPES (pH 7.4) containing [^{14}C]inulin (1.0 uCi/ml, specific activity 2.4 mCi/g) and unlabeled inulin to bring the final inulin concentration to 1.08 mM. The lipid was dispersed by vortexing and the suspension was allowed to swell for 2 hours. Unsequestered [^{14}C]inulin was removed and tritiated water was added following the procedures described above for MPVs.

One milliliter samples of the sixteen preparations (a high concentration EPC, a low concentration EPC, a high concentration SPC, and a low concentration SPC for SPLVs, FATMLVs, MPVs and MLVs) were placed in 30 ml Corex tubes and dried in a dessicator for 2 days at room temperature under high vacuum using the vacuum pump described in Example 2. Another set of samples were lyophilized. Specifically, 1 ml samples were frozen using the shell freezing technique in 30 ml Corex tubes and then dried overnight in a model FDX-1-84-D Flexi-dry lyophilization unit (FTS Systems, Inc., Stone Ridge, New York). Control samples (1 ml in 30 ml Corex tubes) were covered and left at room temperature for 2 days.

Percent inulin retention and percent residual water were determined following the procedures and using the equipment described in Example 2. The results are shown in Table 1.

A comparison of the inulin retention values for the various preparations reveals that: 1) formulations having a high phospholipid concentration prior to dehydration suffer less damage (i.e., less leakage of the internal contents of the liposomes) than formulations having a low phospholipid concentration; 2) egg PC vesicles generally suffer less damage than soy PC vesicles; and 3) MPVs generally suffer less damage than SPLVs and FATMLVs. In addition, the data shows that freezing of the preparation prior to dehydration (the "lyophilization" experiments) results in significantly more damage to the vesicles than does dehydration without prior freezing (the "vacuum dessication" experiments), irrespective of vesicle type, lipid type, or lipid concentration.

TABLE 1

Inulin Retention as a Function of Vesicle Type, Lipid Type and Lipid Concentration			
Lipid Concentration	Vacuum Dessication	Lyophilization	Control
MPV			
EPC (low)	47.0 ± 0.5	27.1 ± 3.1	86.6 ± 6.1
EPC (high)	63.9 ± 6.0	58.3 ± 1.7	101.3 ± 1.8
SPC (low)	41.0 ± 3.5	29.2 ± 5.3	71.7 ± 10.1
SPC (high)	54.6 ± 5.4	42.4 ± 1.8	96.9 ± 2.2
MLV			
EPC (low)	44.6 ± 14.7	40.7 ± 24.6	N.D.
EPC (high)	62.0 ± 3.8	55.4 ± 5.8	99.8 ± 2.1
SPC (low)	28.4 ± 10.1	18.3 ± 3.9	N.D.
SPC (high)	60.4 ± 2.2	53.8 ± 3.3	98.4 ± 7.8
SPLV			
EPC (low)	31.3 ± 0.6	17.0 ± 3.8	69.3 ± 7.5
EPC (high)	56.4 ± 1.9	54.4 ± 3.5	98.5 ± 2.8
SPC (low)	37.2 ± 2.9	24.6 ± 0.2	87.6 ± 1.0
SPC (high)	48.4 ± 6.2	44.6 ± 2.3	97.8 ± 1.7
FATMLV			
EPC (low)	35.6 ± 4.2	18.4 ± 2.6	102.1 ± 4.9
EPC (high)	54.9 ± 0.1	34.8 ± 7.7	95.3 ± 0.4
SPC (low)	24.6 ± 12.2	24.4 ± 1.6	89.8 ± 11.4
SPC (high)	43.7 ± 6.0	27.9 ± 0.7	96.1 ± 1.4
Values reported = % of original inulin retained in the preparation after dehydration and rehydration ± S.D. N.D. = Not determined. Low lipid concentration = 3 umol lipid hydrated with 1 ml prior to dehydration. High lipid concentration = 80 umol lipid hydrated with 1 ml prior to dehydration. Drying Time - Vacuum Dessication -- 2 days - Lyophilization -- 1 day (residual water levels after dehydration equaled approximately 2% or less for both procedures) Number of experiments per data point = 2; data corrected for blank, 2 channel spillover; EPC = Egg PC; SPC = Soy PC.			

Claims

1. A method for dehydrating liposome comprising the steps of (a) preparing a liposome preparation composed of liposomes which have multiple lipid layers and (b) removing water from the preparation without prior freezing of the preparation, said water being removed to an end point which results in sufficient water being left in the preparation so that the integrity of a substantial portion of the multiple lipid layers is retained upon rehydration, that amount of residual water left in the preparation being at least about 2% of water of lipid and more preferably from between about 2% and 5% of water.

2. The method for dehydrating liposomes according to claim 1 comprising the steps of: (a) preparing a liposome which includes one or more protective sugars ; and (b) removing water from the prepartion, wherein said sugars are present at protective concentration.

3. A method according to claim 1 wherein the concentration of sugar is at least 5 moles of sugar per mole of phospholipid.

4. The method of claim 1 wherein the one or more protective sugars are disaccharide sugars.

5. The method of claim 1 wherein the one or more protective sugars are selected from the group consisting of trehalose, maltose, sucrose, glucose, lactose and dextran.

6. The method of claim 1 wherein the one or more protective sugars are aminoglycosides.

7. The method of claim 6 wherein the sugar is streptomycin or dihydrostreptomycin.

8. Liposome preparation which is a dehydrated liposome prepared in accordance with the method of any of claims 1 to 7.

9. The liposome preparation of claim 8, wherein the internal contents comprise a drug.

10. The liposome preparation of claim 8 wherein the drug comprises an antineoplastic agent.

11. The liposome preparation of claim 8 wherein the antineoplastic agent comprises doxorubicin.

12. The liposome preparation of claim 8, wherein the internal content does not comprise a drug.

Patentansprüche

1. Verfahren zur Dehydratisierung von Liposomen, umfassend die folgenden Schritten: (a) Herstellung eines Liposomenpräparats, das aus Liposomen mit mehrfachen Lipidschichten besteht; und (b) Entzug von Wasser aus dem Präparat ohne vorheriges Einfrieren des Präparats, wobei das Wasser bis zu einem Endpunkt entfernt wird, bei dem noch genugend Wasser im Präparat verbleibt, so daß die Unversehrtheit eines wesentlichen Anteils der multiplen Lipidschichten nach der Rehydratisierung erhalten bleibt, wobei die im Präparat verbleibende Restwassermenge mindestens etwa 2 % und bevorzugterweise zwischen etwa 2 und 5 % Wasser beträgt.

2. Verfahren zur Dehydratisierung von Liposomen nach Anspruch 1, umfassend die folgenden Schritten: (a) Herstellung eines Liposoms, das einen oder mehrere Schutzzucker enthält; und (b) Entzug von Wasser aus dem Präparat, wobei die besagten Zucker in schützender Konzentration vorhanden sind.

3. Verfahren nach Anspruch 1, wobei die Zuckerkonzentration mindestens 5 Mol Zucker pro Mol Phospholipid beträgt.

4. Verfahren nach Anspruch 1, wobei der bzw. die Schutzzucker Disaccharidzucker sind.

5. Verfahren nach Anspruch 1, wobei der bzw. die Schutzzucker aus der Gruppe Trehalose, Maltose, Saccharose, Glukose, Laktose und Dextran ausgewählt werden.

6. Verfahren nach Anspruch 1, wobei der bzw. die Schutzzucker Aminoglykoside sind.

7. Verfahren nach Anspruch 6, wobei Zucker Streptomycin oder Dihydrostreptomycin ist.

8. Liposomenpräparat, bestehend aus dehydratisierten Liposomen, die nach dem Verfahren eines der Anspruche 1 bis 7 hergestellt wurden.

9. Liposomenpräparat nach Anspruch 8, in dem der innere Gehalt ein Arzneimittel enthält.

10. Liposomenpräparat nach Anspruch 8, in dem das Arzneimittel ein antineoplastisches Mittel enthält.

11. Liposomenpräparat nach Anspruch 8, in dem das antineoplastische Mittel Doxorubicin enthält.

12. Liposomenpräparat nach Anspruch 8, dadurch gekennzeichnet, daß der innere Gehalt kein Arzneimittel enthält.

Revendications

1. Méthode de déshydratation des liposomes comprenant les étapes suivantes : (a) la préparation d'une préparation de liposomes se composant de liposomes qui ont des couches lipidiques multiples, et (b) le retrait de l'eau de la préparation sans congélation préalable de la préparation, ladite eau étant retirée jusqu'à un point final où il reste une quantité suffisante d'eau dans la préparation de manière à ce que l'intégrité d'une partie importante des couches lipidiques multiples soit retenue lors de la réhydratation, la quantité d'eau résiduelle laissée dans la préparation étant d'au moins 2 % environ de l'eau du lipide et, plus préférablement, entre 2 et 5 % environ d'eau.

2. La méthode de déshydratation des liposomes conformément à la revendication 1 comprenant les étapes suivantes : (a) préparation d'un liposome qui comprend un ou plusieurs sucres protecteurs ; et (b) retrait de l'eau de la préparation, lesdits sucres étant présents à la concentration protectrice.

3. Méthode selon la revendication 1 où la concentration de sucre est d'au moins 5 moles de sucre par mole de phospholipide.

4. La méthode de la revendication 1 où le ou les sucres protecteurs sont des sucres disaccharides.

5. La méthode de la revendication 1 où le ou les sucres protecteurs sont choisis à même le groupe se composant de tréhalose, maltose, sucrose, glucose, lactose et dextrane.

6. La méthode de la revendication 1 où le ou les sucres protecteurs sont des aminoglycosides.

7. La méthode de la revendication 6 où le sucre est la streptomycine ou la dihydrostreptomycine.

8. Préparation de liposomes consistant en liposome déshydraté préparé conformément à la méthode de l'une quelconque des revendications 1 à 7.

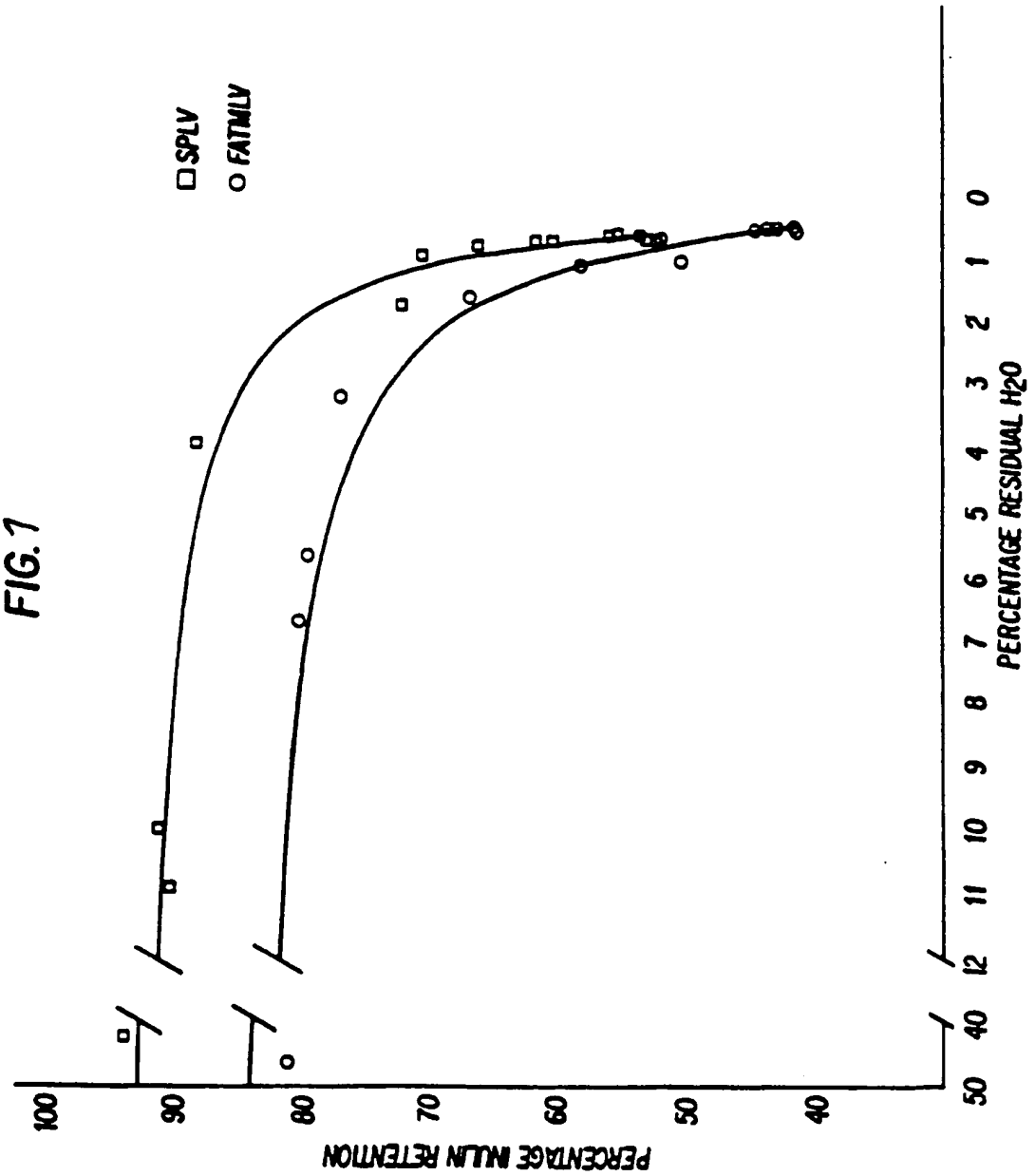
9. La préparation de liposome de la revendication 8 dont le contenu interne comprend un médicament.

10. La préparation de liposome de la revendication 8 dont le médicament comprend un agent antinéoplasique.

11. La préparation de liposome de la revendication 8 dont l'agent antinéoplasique comprend de la doxorubicine.

12. La préparation de liposome de la revendication 8 dont le contenu interne ne comprend pas un médicament.

FIG. 1


□ SPLV
○ FATMILV
PERCENTAGE INULIN RETENTION
100
90
80
70
60
50
40
50
40
12
11
10
9
8
7
6
5
4
3
2
1
0
PERCENTAGE RESIDUAL H2O